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Acetylation of FoxO1 Activates Bim Expression to Induce Apoptosis in Response to Histone Deacetylase Inhibitor Depsipeptide Treatment[1,2]

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Abstract

Histone deacetylase (HDAC) inhibitors have been shown to induce cell cycle arrest and apoptosis in cancer cells. However, the mechanisms of HDAC inhibitor induced apoptosis are incompletely understood. In this study, depsipeptide, a novel HDAC inhibitor, was shown to be able to induce significant apoptotic cell death in human lung cancer cells. Further study showed that Bim, a BH3-only proapoptotic protein, was significantly upregulated by depsipeptide in cancer cells, and Bim's function in depsipeptide-induced apoptosis was confirmed by knockdown of Bim with RNAi. In addition, we found that depsipeptide-induced expression of Bim was directly dependent on acetylation of forkhead box class O1 (FoxO1) that is catalyzed by cyclic adenosine monophosphate–responsive element-binding protein–binding protein, and indirectly induced by a decreased four-and-a-half LIM-domain protein 2. Moreover, our results demonstrated that FoxO1 acetylation is required for the depsipeptide-induced activation of Bim and apoptosis, using transfection with a plasmid containing FoxO1 mutated at lysine sites and a luciferase reporter assay. These data show for the first time that an HDAC inhibitor induces apoptosis through the FoxO1 acetylation-Bim pathway.

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Introduction

Bim is a proapoptotic BH3 domain-only member of the Bcl-2 family, which is required for hematopoietic cell homeostasis and acts as a barrier against autoimmune disease [1]. Recently, it has been reported that Bim is involved in the regulation of apoptosis in many different types of cells [2–7]. The apoptotic activity of Bim was thought to be mediated through several possible mechanisms including activation of Bax or Bak [3,4,8]. Bim's expression is also tightly regulated by Rb-E2F1 [9], phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) [10], or the extracellular signal–regulated kinase/mitogen-activated protein kinase pathway [11,12].

The forkhead box transcription factor, class O (FoxO), is a mammalian homolog of DAF-16, which is known to regulate life span in *Caenorhabditis elegans* [13,14]. The FoxO factors including FoxO1, FoxO3a, FoxO4, and FoxO6 share DNA-binding specificity to a core consensus site called the forkhead-responsive element [15–19] and regulate the transcription of genes involved in several cellular processes such as cell cycle arrest, apoptosis, and DNA repair in response to oxidative stress, differentiation, or glucose metabolism [10,18,20,21]. Among the FoxO members, FoxO1 activity is negatively regulated by PI3-K/Akt, which phosphorylates FoxO1 at multiple sites and forces FoxO1 into the cytoplasm, and thus, decreases its transcriptional activity [18,21–23]. Recently, cell death induced by FoxO3a was reported to be mediated through Bim, which is one of the FoxO-target genes [23–25]. It was thus important to clarify whether FoxO1 is also involved in the process of Bim-induced apoptosis.

Abbreviations: CBP, cyclic adenosine monophosphate–responsive element-binding protein–binding protein; FHL2, four-and-a-half LIM-domain protein 2; FoxO1, forkhead box class O1; HDAC inhibitor, histone deacetylase inhibitor; PI3-K, phosphatidylinositol 3-kinase; PKB/Akt, protein kinase B; SAHA, suberoylanilide hydroxamic acid; SIRT1, sirtuin 1

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Posttranslational modification of FoxOs, and especially phosphorylation, is considered to play an important role in activating Bim [25,26]. For example, saturated free fatty acids were reported to induce dephosphorylation of FoxO3a and in turn induce the expression of the intracellular death mediator Bim [25]. In addition, atorvastatin, an antioxidant reagent, prevents H_2O_2-induced apoptosis by increasing phosphorylation of FoxO4 and thus reducing the expression of Bim in endothelial progenitor cells [26]. Other posttranslational modifications such as acetylation have also been identified. The acetylation status of FoxOs is regulated by a balance between proteins with histone acetylase activity and proteins with histone deacetylase (HDAC) activity. Cyclic adenosine monophosphate–responsive element-binding protein–binding protein (CBP) and p300 have been found to be able to induce acetylation of FoxO proteins [14,18,27]. In addition to acetylases, proteins with HDAC activity such as sirtuin 1 (SIRT1) or a four-and-a-half LIM-domain protein 2 (FHL2) have been reported to be involved in the acetylation of FoxOs and the expression of Bim [27–29]. Thus, it is of value to clarify whether acetylation of FoxO1 is involved in HDAC inhibitors–induced apoptosis.

Histone deacetylase inhibitors have been extensively studied and have been used as potential therapeutic agents for tumors including leukemia [30–35]. Histone deacetylase inhibitors are also reported to induce acetylation of nonhistone proteins [36,37] and to have the ability to acetylate hyperacetylated nucleosome core histones [30] or to demethylate DNA [38]. The importance of HDAC inhibitors in clinical therapy is based on the ability of almost all HDAC inhibitors to induce a range of antitumor activities including induction of apoptosis [30,39–41]. Several HDAC inhibitors were reported to induce apoptosis in tumor cells by increasing the expression of Bim or other related genes [9,42–47]. For example, Bim was reported to play an important role in the apoptotic and therapeutic activities of HDAC inhibitors on the basis of a mouse model of B-cell lymphoma [46]. Histone deacetylase inhibitors were also found to induce apoptosis of tumor cells from patients with chronic lymphocytic leukemia through Bim and Noxa [47]. However, although HDAC inhibitors show activity in the induction of Bim-associated apoptosis, the exact mechanisms underlying this effect are not well understood.

In this study, human lung cancer cell lines H460, H719, A549, and H1299 were treated with the HDAC inhibitor depsipeptide to explore the mechanism of depsipeptide-induced apoptosis. We found that Bim plays a pivotal role in depsipeptide-induced apoptosis in some of these cells. Furthermore, FoxO1 acetylation was confirmed to be required for the activation of Bim, which is catalyzed by CBP, and a weakened association of SIRT1 and FoxO1 in response to depsipeptide treatment.

Materials and Methods

Cell Lines and Cell Culture

Human lung cancer cell lines A549, H1299, NCI-H460, and H719 were grown in RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 45 minutes) and an appropriate amount of penicillin/streptomycin in a 37°C incubator with a humidified 5% CO_2 atmosphere.

Reagents

The HDAC inhibitor depsipeptide was kindly provided by the National Institutes of Health and was dissolved in DMSO to a stock concentration of 50 μM and stored at 4°C. The HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) and apicidin were purchased from Sigma (St. Louis, MO).

Flow Cytometry Assay

The DNA content of cultured cells was analyzed by flow cytometry assay as previously described [48]. Apoptotic cells have a lower DNA content than that in normal cells and appear as a pre-G_1 peak on a DNA cell cycle histogram, and cells were harvested to quantitate the amount of apoptosis present after various treatments. In brief, cells were trypsinized and washed once with cold phosphate-buffered saline. Cells were then fixed with 70% ethanol and stored overnight at –20°C. Propidium iodide (10 μg/ml; Sigma) was added to stain cells in the presence of RNase (Promega, Madison, WI) at 37°C for 10 minutes, and cells were then analyzed on a FACscan Flow Cytometer with manual gating using CellQuest software.

Colony Formation Assay

A549 and H1299 cells were treated with different doses of depsipeptide, and appropriate cell numbers were plated in triplicate into six-well plates for survival determination. Cells were incubated for 10 to 14 days at 37°C incubator to allow for colony formation. Colonies (a colony was defined as containing >50 cells) were then fixed with methanol/acetic acid and stained with crystal violet.

Reverse Transcription–Polymerase Chain Reaction

Primers for polymerase chain reaction (PCR) were designed as follows: Bim-F, 5′-GAGAAGGTAGACAATTGCAG-3′, Bim-R, 5′-GACAATGTAACGTAACAGTCG-3′; FoxO1-F, 5′-CTCCCATACCCACCCTG-3′, FoxO1-R, 5′-AATGAACATGCCATCCAAG-3′; FHL2-F, 5′-GCCAAGAAGTGTGCTGGG-3′, FHL2-R, 5′-GCAACGGGAGGTTACAGAG-3′; SIRT1-F, 5′-CCTGACTTCAGATCAAGAGACGGTA-3′, SIRT1-R, 5′-CTGATTAAAAATGTCTCCACGAACAG-3′; p27-F, 5′-TGGAGAAGCACTGCAGAGAC-3′, p27-R, 5′-GCGTGTCCTCAGAGTTAGCC-3′; GADD45-F, 5′-CCATGCAGGAAGGAAAACTATG-3′, GADD45-R, 5′-CCCAAACTATGGCTGCACACT-3′; CBP-F, 5′-GGGCCTGTCATCAACACCC-3′, CBP-R, 5′-CGTAGTCCTCGCACACAGTG-3′. The PCR products were run on 2% agarose gels, stained with ethidium bromide, and evaluated with UV light.

Plasmids and Small Interfering RNA Transfection

Plasmids used for transfection in this study included the mutant FoxO1 with acetylation sites 3KR in which lysine (Lys-242, Lys-245, and Lys-262) was replaced by arginine (gifts from Akiyoshi Fukamizu) and wild-type FoxO1 (purchased from Addgene). The Bim small interfering RNA (siRNA) sequence was designed as 5′-CAAUUGUCUACCUUCUCGG-3′, and the nonspecific siRNA (control) sequence was designed as 5′-GACCACGAGUAAAAGUAGU-3′ [49]. The FoxO1 siRNA sequence was designed as 5′-GAGCGTGCCCTACTTCAAG-3′, and the nonspecific siRNA (control) sequence was designed as 5′-CCACTACCTGAGCACCCAG-3′ [50]. The FoxO3a siRNA sequence was designed as 5′-GAGCUCUUGGUGGAUCAUCTT-3′ [51]. The FHL2 siRNA sequence was designed as 5′-UCUCUCUUUGGCAAGAAGU-3′ [52]. The CBP siRNA sequence was designed as 5′-TAGTAACTCTGGCCATAGC-3′ [53]. These RNAi oligonucleotides were purchased from Genechem (Shanghai, China). After transfection, the cells were harvested and subjected to Western blot analysis, reverse transcription–PCR (RT-PCR), or Luciferase assay.

Luciferase Assay

H1299 cells were plated on 24-well plates after treatment and were incubated until 60% to 80% confluency was reached. Cells were transfected with a Bim-pGL3 luciferase reporter construct (kindly provided by Qiang Yu) [9] using the GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). After transfection, cells were incubated in the presence or absence of depsipeptide for 12 hours. Luciferase activity in cell lysates was measured with the Luciferase assay system (Promega) and normalized for protein in the cell lysate. The results are the relative luciferase activity of the treated cells over that of the control cells. All transfection experiments were carried out in triplicate wells and were repeated separately at least three times.

Western Blot Analysis

Protein expression was detected by Western blot analysis as previously described with minor modifications [54]. Equal amounts of proteins (100 to 150 μg) were size fractionated by 9 to 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Anti-Bim (H-191; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax (sc-6236; Santa Cruz Biotechnology), anti-FoxO1 (Cell Signaling Technology, Beverly, MA), anti-FoxO3a (Cell Signaling), anti-FHL2 (Santa Cruz Biotechnology), anti-SIRT1 (Santa Cruz Biotechnology), p300 (H-272; Santa Cruz Biotechnology), CBP (A-22; Santa Cruz Biotechnology), P300/CREB binding protein (CBP)–associated factor (PCAF) (C-16; Santa Cruz Biotechnology), anti–β-actin (Santa Cruz Biotechnology), and anti–acetyl-lysine (Upstate Biotechnology, Lake Placid, NY) were used, and the blots were developed using an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Chromatin Immunoprecipitation Assay

H1299 cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C and then washed with cold phosphate-buffered saline. The cell pellet was resuspended in lysis buffer, followed by sonication to an average DNA length of 500 to 1000 bp. Antibodies were added to each of the samples, which were then rotated at 4°C overnight. After interaction with protein A beads and incubation overnight at 65°C to reverse the cross-links, the DNA was dissolved in Tris-EDTA buffer and analyzed by PCR. The anti-FoxO1 antibodies were added separately into the reaction solutions. Primers used for PCR were as follows: Bim: 5′-AGCAAGCAGAGTTACTCCGGTAAACA-3′ (sense) and 5′-CCCGCTCCTACGCCCAATCA-3′ (antisense); p27: 5′-GTCCCTTCCAGCTGTCACAT-3′ (sense) and 5′-GGAAACCAACCTTCCGTTCT-3′ (antisense).

Coimmunoprecipitation

A549 cells were harvested and then lysed in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, 0.05% SDS, 1 mM PMSF, and a 1% cocktail of protease inhibitors) on ice for 20 minutes. After centrifugation at 4°C at 13,000 rpm for 10 minutes, antibodies were added to the supernatant on ice for 1 hour. Agarose G was then added, and the samples were rolled at 4°C for 1 hour. After the beads were washed three times with lysis buffer, the pellets were dissolved into 2× SDS loading buffer after centrifugation. The protein was analyzed by Western blot analysis with different antibodies.

Data Analysis

Statistical analysis was performed to assess the difference between the two groups under multiple conditions by one-way analysis of variance using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results

Depsipeptide Induces Apoptosis in Human Lung Cancer Cells

In this study, human lung cancer cell lines H460, A549, H719, and H1299 cells were exposed to depsipeptide, and depsipeptide-induced cytotoxicity was then evaluated. First, flow cytometry was performed to analyze changes in cell cycle and cell death in these cells in response to depsipeptide treatment. The cells with DNA content smaller than G_0/G_1 (designated pre-G_1 peak on the DNA histogram) were considered to be apoptotic cells. Although depsipeptide induced a G_2 arrest in all cell lines tested (data not shown), depsipeptide-induced apoptotic cell death was cell line–dependent. For example, depsipeptide showed a dose-dependent cytotoxicity to H460, A549, and H719 cells (Figure 1*A*). Conversely, depsipeptide was not able to induce apoptosis in H1299 cells (Figure 1*A*). Moreover, the change in poly(ADP-ribose) polymerase (PARP) cleavage (from 116 kDa cleaved to 89 kDa), which is thought to be a late event in apoptosis, was observed in depsipeptide-treated A549 cells assayed with Western blot analysis. As shown in Figure 1*B*, a dose-dependent increase in cleaved 89-kDa PARP fragments was observed when A549 cells were treated with depsipeptide, but no cleaved PARP fragments were found in H1299 cells. To further confirm the effect of depsipeptide induced cell killing, a colony formation assay was performed. A549 and H1299 cells were treated with depsipeptide at different doses and plated into six-well plates for colony formation. Figure 1*C* shows that depsipeptide induced a significant decrease in colony formation in A549 cells compared with that in H1299 cells. These data showed that depsipeptide-induced apoptosis of human lung cancer cells was cell line–dependent. In addition, to evaluate whether A549 cells were sensitive to other HDAC inhibitors, SAHA and apicidin were also used to perform these experiments. As shown in Figure 1*D*, although all HDAC inhibitors tested induced apoptosis in A549 cells, depsipeptide was much more effective in inducing apoptosis than other HDAC inhibitors (44.9 ± 1.3% cell apoptosis in depsipeptide-treated cells compared with 6.8 ± 0.5% cell apoptosis in SAHA-treated cells or with 11.7 ± 2.1% cell apoptosis in apicidin-treated cells), indicating that depsipeptide is a potent inducer of apoptosis in A549 cells.

Activation of Bim Is Responsible for Depsipeptide-Induced Apoptosis

It has been reported that Bcl-2 family members are involved in various reagent-induced apoptosis [55–57]. To explore the effect of Bcl-2 family members on depsipeptide-induced apoptosis, the expression of Bcl-2 family proteins including Bcl-2, Bax, and Bim was evaluated by Western blot analysis. There are three major isoforms of Bim created by alternative splicing, namely, BimEL, BimL, and BimS, and only BimEL was readily identifiable by Western blot analysis in our study. As shown in Figure 2*A*, expression of BimEL (hereafter referred to as Bim) was significantly increased in a dose-dependent manner. For example, the expression of Bim was increased by 7.0- or 21.0-fold in A549 cells treated with depsipeptide at 0.05 or 0.1 μM for 24 hours (Figure 2*A*). However, the expression of Bcl-2 and Bax was not obviously changed in these cells under identical treatment conditions (Figure 2*A*). Next, RT-PCR was performed to investigate whether activation of Bim results from transcriptional regulation in A549 cells induced by depsipeptide treatment. As shown in Figure 2*B*, Bim mRNA

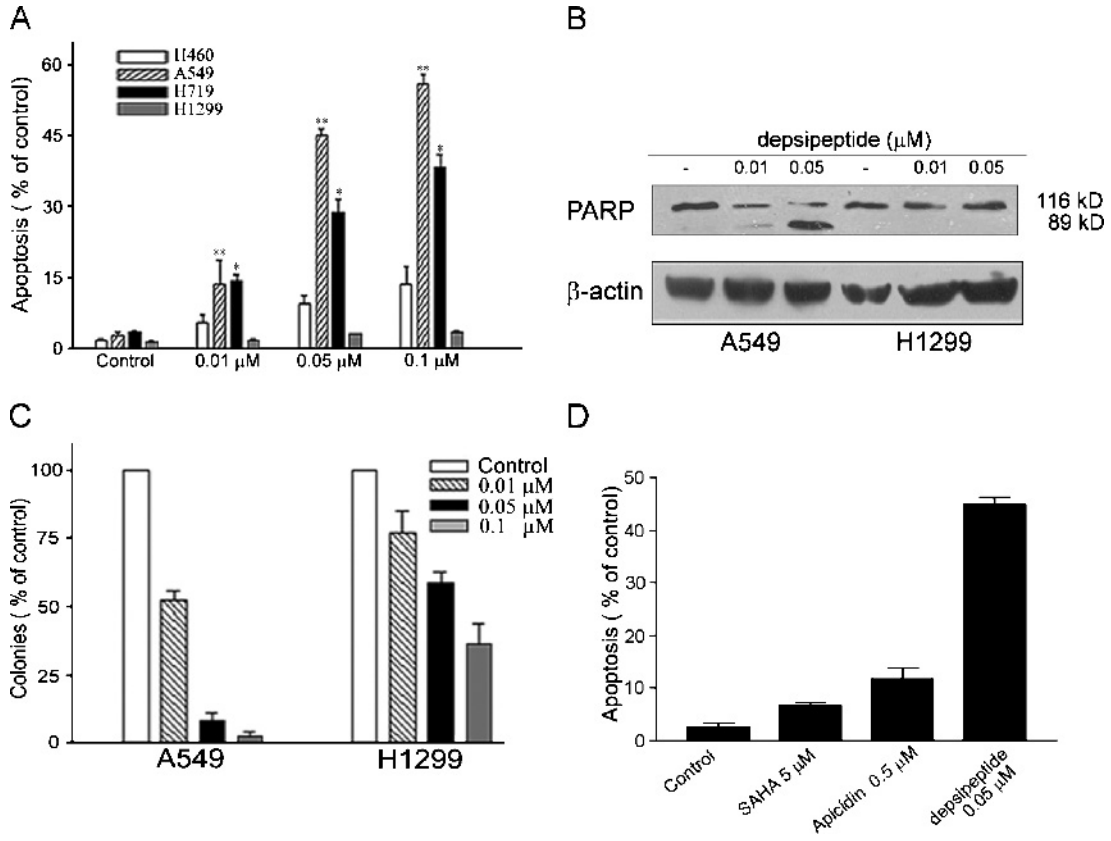

Figure 1. Depsipeptide induces apoptosis in human lung cancer cell lines. (A) Human lung cancer cell lines H460, A549, H719, and H1299 were treated with or without depsipeptide over a range of 0.01 to 0.1 μM for 24 hours, and cells were then harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Apoptotic cells were evaluated and presented as a percentage of total cells. *$P < .05$, **$P < .01$. (B) A549 and H1299 cells were treated with depsipeptide (0.01 or 0.05 μM) for 24 hours. Cells were then harvested, and protein was extracted for Western blot analysis to evaluate for PARP cleavage. β-Actin is shown as a loading control. (C) A549 and H1299 cells were treated with various doses of depsipeptide (0.01 to 0.1 μM) and then plated into six-well plates for colony formation. After 10 to 14 days, colonies were fixed with methanol/acetic acid, stained with crystal violet, and counted under a microscope. (D) A549 cells were also treated with or without the HDAC inhibitors SAHA (5 μM) or apicidin (0.5 μM) for comparison to depsipeptide treatment (0.05 μM) for 24 hours, and cells were then harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Results are presented as the mean ± SD of three experiments performed in triplicate for flow cytometry analysis or colony formation. Statistical analysis was carried out with one-way analysis of variance.

was obviously increased after depsipeptide treatment. For instance, depsipeptide (0.05 or 0.1 μM for 24 hours) induced a three- or nine-fold increase in Bim mRNA. These results suggested that depsipeptide induced expression of Bim at both protein and mRNA levels. However, the expression of neither Bim protein nor mRNA was affected by depsipeptide treatment in H1299 cells (Figure 2, *C* and *D*). These findings were consistent with the apoptotic results described above (Figure 1, *A*–*C*) and suggested that Bim may play a major role in inducing apoptosis in A549 cells in response to depsipeptide treatment.

To further confirm this observation, A549 cells were transiently transfected with siRNA against Bim or a nonspecific siRNA (named as control RNAi) and then treated with depsipeptide. Twenty-four hours after treatment, cells were harvested and subjected to RT-PCR or flow cytometry assay. As shown in Figure 2*E*, depsipeptide (0.05 μM for 24 hours) induced a six-fold increase in Bim mRNA compared with that in control RNAi–transfected cells, and the expression of Bim induced by depsipeptide was effectively reduced after Bim RNAi transfection. Similarly, the numbers of apoptotic cells after depsipeptide treatment in A549 cells decreased after transfection with Bim siRNA as assayed with flow cytometry (Figure 2*F*). These data suggest that depsipeptide-induced apoptosis in A549 cells was modulated by the proapoptotic protein Bim.

Depsipeptide Induces an Increase in FoxO1 Expression

It is known that Bim is one of the downstream targets of FoxOs [18,21], and evidence has shown that its expression is mediated by FoxO3 [20,23,24]. We thus investigated whether depsipeptide-induced expression of Bim is also mediated through activation of the FoxO family. However, as shown in Figure 3*A*, the expression of FoxO3a was not obviously increased after depsipeptide treatment. To further exclude FoxO3a activation as the mechanism of depsipeptide-induced apoptosis, FoxO3a siRNA or a control RNAi was delivered into A549 cells, which were then subjected to depsipeptide treatment. As shown in Figure 3*B*, the expression of FoxO3a protein was effectively reduced by 90% after transfection with FoxO3a siRNA compared with that in control RNAi–transfected A549 cells. It was of interest that depsipeptide-induced apoptosis was not decreased in A549 cells with FoxO3a knockdown compared with that in control RNAi–transfected cells as evaluated with flow cytometry (Figure 3*B*), demonstrating that depsipeptide-induced apoptosis was FoxO3a-independent. Conversely,

the FoxO1 expression was significantly increased in the depsipeptide-treated A549 cells (Figure 3*C*). This depsipeptide-induced alteration of FoxO1 expression resulted from transcriptional regulation, in which depsipeptide was able to induce a significant increase in expression of FoxO1 mRNA (three-fold increase in depsipeptide-treated cells compared with untreated cells; Figure 3*D*), showing that depsipeptide-induced apoptosis was FoxO1-dependent.

In addition, to investigate whether the FoxO1/Bim pathway is unique to depsipeptide-induced apoptosis, A549 cells were exposed to the HDAC inhibitors SAHA and apicidin. As shown in Figure 3*E*, there were no obvious changes in mRNA levels of Bim or FoxO1, suggesting strongly that the FoxO1/Bim pathway is not involved in SAHA- or apicidin-induced apoptosis in A549 cells.

FoxO1 Mediates Bim Expression in Response to Depsipeptide Treatment

In view of the fact depsipeptide induced an increase in expression of both FoxO1 and Bim, we were led to consider whether there is a causal relation between the increase of FoxO1 and the expression of Bim in response to depsipeptide treatment. We thus tested whether FoxO1 expression is required for subsequent Bim expression in depsipeptide-treated cells. A plasmid with wild-type FoxO1 tagged with a green fluorescent protein (GFP) was transiently transfected into H1299 cells and then treated with depsipeptide (endogenous FoxO1 showed only very low expression in H1299 cells). As shown in Figure 4*A*, depsipeptide induced a significant expression of FoxO1 (three-fold increase compared with that in cells not treated with depsipeptide after transfection with GFP-FoxO1). Accompanying this expression of FoxO1, the expression of Bim was also significantly increased in response to depsipeptide treatment (Figure 4*A*). Consequently, depsipeptide-induced apoptosis was observed only in cells transfected with the GFP-FoxO1 as assayed with flow cytometry (Figure 4*B*).

To further clarify these results, A549 cells were transiently transfected with FoxO1 siRNA or control RNAi and were then treated with depsipeptide. As shown in Figure 4*C*, the expression of FoxO1 was reduced by 70% after FoxO1 siRNA transfection. Similarly, Bim expression was also significantly reduced in the FoxO1 siRNA–treated cells compared with that in control RNAi–treated cells in response to depsipeptide

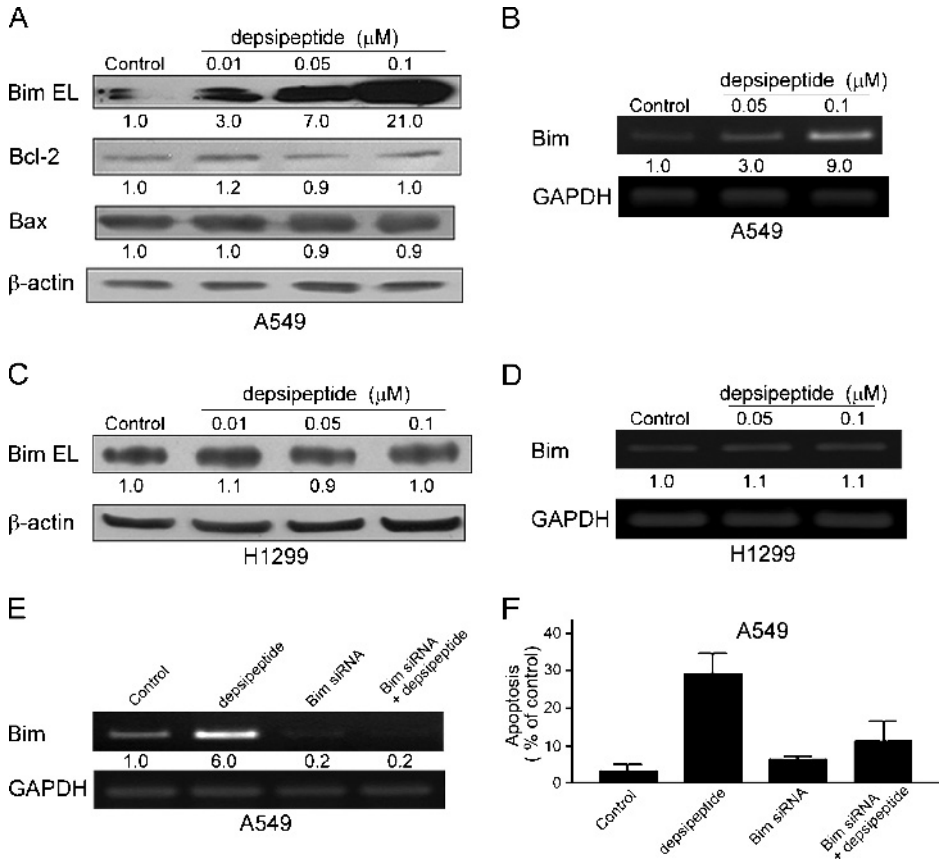

Figure 2. Activation of Bim is responsible for depsipeptide induced apoptosis. A549 (A) or H1299 cells (C) were treated with depsipeptide (0.01 to 0.1 μM for 24 hours). Cells were then harvested for Western blot analysis. Specific antibodies were used to evaluate the expression of Bim, Bcl-2, or Bax. β-Actin is shown as a loading control. A549 (B) or H1299 cells (D) were treated with depsipeptide (0.05 or 0.1 μM for 24 hours), and RNA was then extracted for RT-PCR assay to identify changes in Bim mRNA. GAPDH was used as a loading control for RT-PCR. (E) A549 cells were transiently transfected with oligonucleotides of Bim siRNA or a control RNAi and then treated with depsipeptide (0.05 μM for 24 hours). The cells were then harvested and subjected to RT-PCR to detect Bim expression. GAPDH was used as a loading control for RT-PCR. (F) After transient transfection with oligonucleotides of Bim siRNA or a control RNAi and treatment with or without depsipeptide (0.05 μM for 24 hours), A549 cells were harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. For all Western blot analysis, β-actin is shown as a loading control. Protein and mRNA bands were scanned by phosphorimaging, and the relative band intensities were normalized to each β-actin or GAPDH band. The band intensity of untreated sample was set as 1. The numerical value of each sample represents the percentage of band intensity relative to that of the untreated sample.

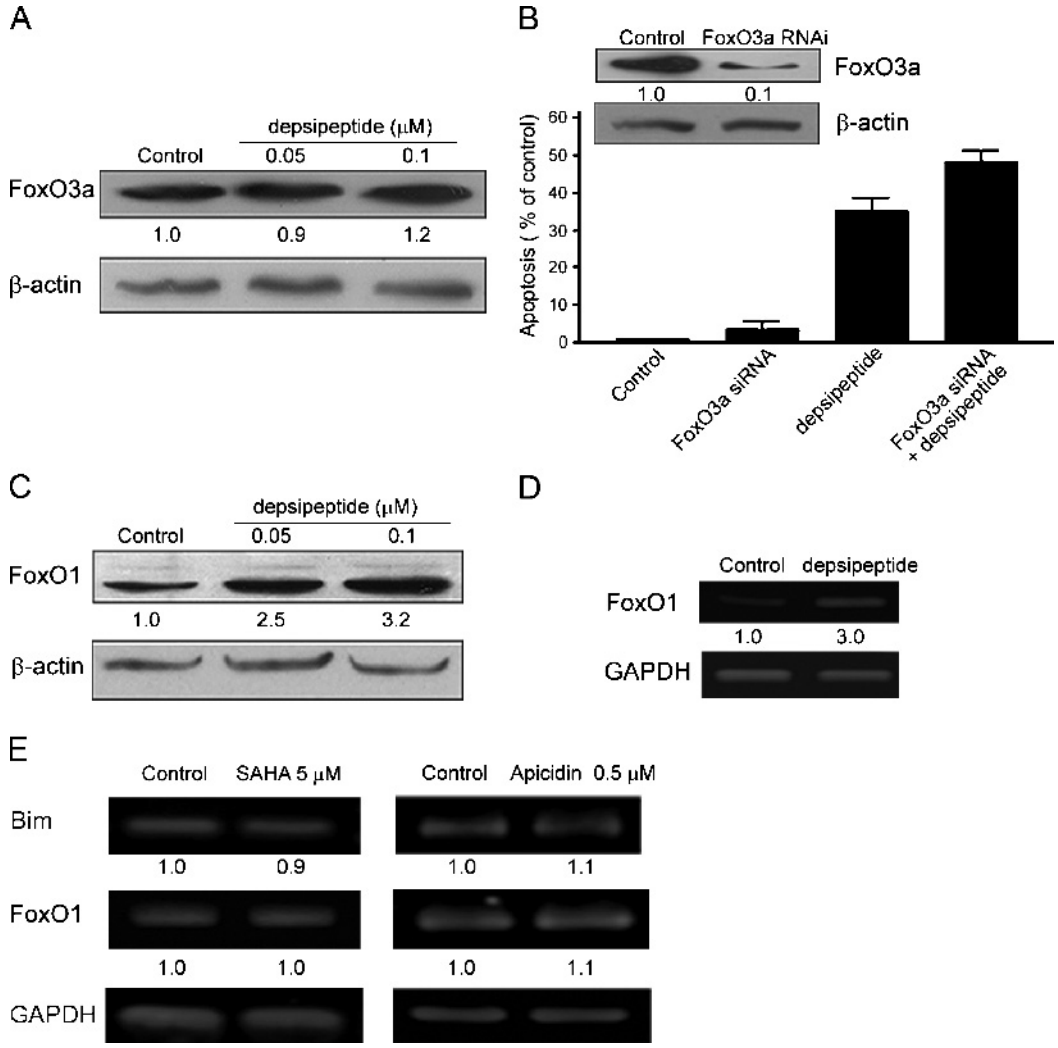

Figure 3. Depsipeptide induces an increase in FoxO1 expression. (A) A549 cells were treated with depsipeptide (0.05 or 0.1 μM for 24 hours). The cells were then harvested and subjected to Western blot analysis to evaluate FoxO3a expression. (B) A549 cells were transfected with either oligonucleotides of FoxO3a siRNA or control RNAi and were then treated with depsipeptide (0.05 μM for 24 hours). Cells were harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles through flow cytometry or through Western blot analysis to detect FoxO3a expression. Apoptotic cells were calculated and presented as a percentage of total cells. (C) A549 cells were treated with depsipeptide (0.05 or 0.1 μM for 24 hours). Cells were then harvested and subjected to Western blot analysis to evaluate FoxO1 expression. (D) A549 cells were treated with depsipeptide at 0.05 μM for 24 hours, and then mRNA was extracted, and RT-PCR was performed to identify changes in FoxO1 mRNA. GAPDH was used as a loading control for RT-PCR. (E) A549 cells were treated with SAHA (5 μM) or apicidin (0.5 μM) for 24 hours, and mRNA was then extracted for RT-PCR assay to identify changes in Bim or FoxO1 mRNA. GAPDH was used as a loading control for RT-PCR. Results are presented as the mean ± SD of three experiments performed in triplicate for flow cytometry analysis. For each Western blot analysis assay, β-actin is shown as a loading control. Protein and mRNA bands were scanned by phosphorimaging, and the relative band intensities are shown as mentioned above.

treatment. These findings were consistent with resultant apoptosis as determined by flow cytometry (Figure 4*D*). Also, vector-driven FoxO1 siRNA was used to constitutively knockdown the target gene, and a colony formation assay was performed to confirm the effect of FoxO1 on the expression of Bim and apoptosis induced by depsipeptide. As shown in Figure 4*E*, a stable transfection of a FoxO1 RNAi plasmid also significantly decreased the mRNA level of FoxO1 and Bim in A549 cells, and this decrease in FoxO1 and Bim expression was consistent with the decrease in apoptosis noted in response to depsipeptide treatment (data not shown). The rate of colony formation in A549 cells with knockdown of FoxO1 was increased approximately 2.5-fold compared with that in control RNAi cells (Figure 4*F*). Depsipeptide induced a significant decrease in colony formation rate in control RNAi A549 cells compared with that in the FoxO1 knockdown cells (Figure 4*F*). These results clearly indicate that FoxO1 expression is prerequisite for the induction of Bim and apoptosis induced by depsipeptide.

FoxO1 Acetylation Is Induced by an Enhanced Recruitment of CBP to FoxO1 and Is Required for Depsipeptide-Induced Bim Expression

Because the role of phosphorylation in the regulation of FoxOs' activity has been extensively characterized [18,21], we next tested whether depsipeptide induces changes in the phosphorylation of FoxO1 at several key sites including Ser-256. However, no increase in phosphorylation of FoxO1 was found at these sites after depsipeptide treatment (data not shown). Thereafter, acetylation changes in FoxO1 after

depsipeptide treatment were investigated using a coimmunoprecipitation (CoIP) assay. An anti–acetyl-lysine antibody was used for detecting total lysine acetylation of all proteins in depsipeptide-treated A549 cells and was followed by probing with anti-FoxO1 antibody. As shown in Figure 5*A*, a significant increase in FoxO1 acetylation was confirmed in these depsipeptide-treated cells (to evaluate acetylation changes in FoxO1 with depsipeptide treatment, an equal amount of FoxO1 was loaded as a control). To further determine the mechanism of depsipeptide-induced lysine acetylation, a CoIP assay was performed to test the interaction of coactivators (PCAF, CBP, or p300) with FoxO1 in the treated cells. As shown in Figure 5*B*, depsipeptide induced an enhancement of recruitment of CBP to FoxO1 (six-fold increase in depsipeptide-treated cells compared with that in the untreated control). However, depsipeptide did not enhance the interaction of p300 with FoxO1 (Figure 5*B*). In addition, no interaction of PCAF with FoxO1 was found in A549 cells (data not shown).

We next investigated the role of CBP-catalyzed acetylation of FoxO1 in activating Bim. A mutant FoxO1-3KR, in which the CBP-dependent acetylation residues sites (Lys-242, Lys-245, and Lys-262) were replaced by arginine, was used for this portion of the study. H1299 cells were transiently transfected with the mutated plasmid or nonspecific plasmid and were then treated with depsipeptide. As shown

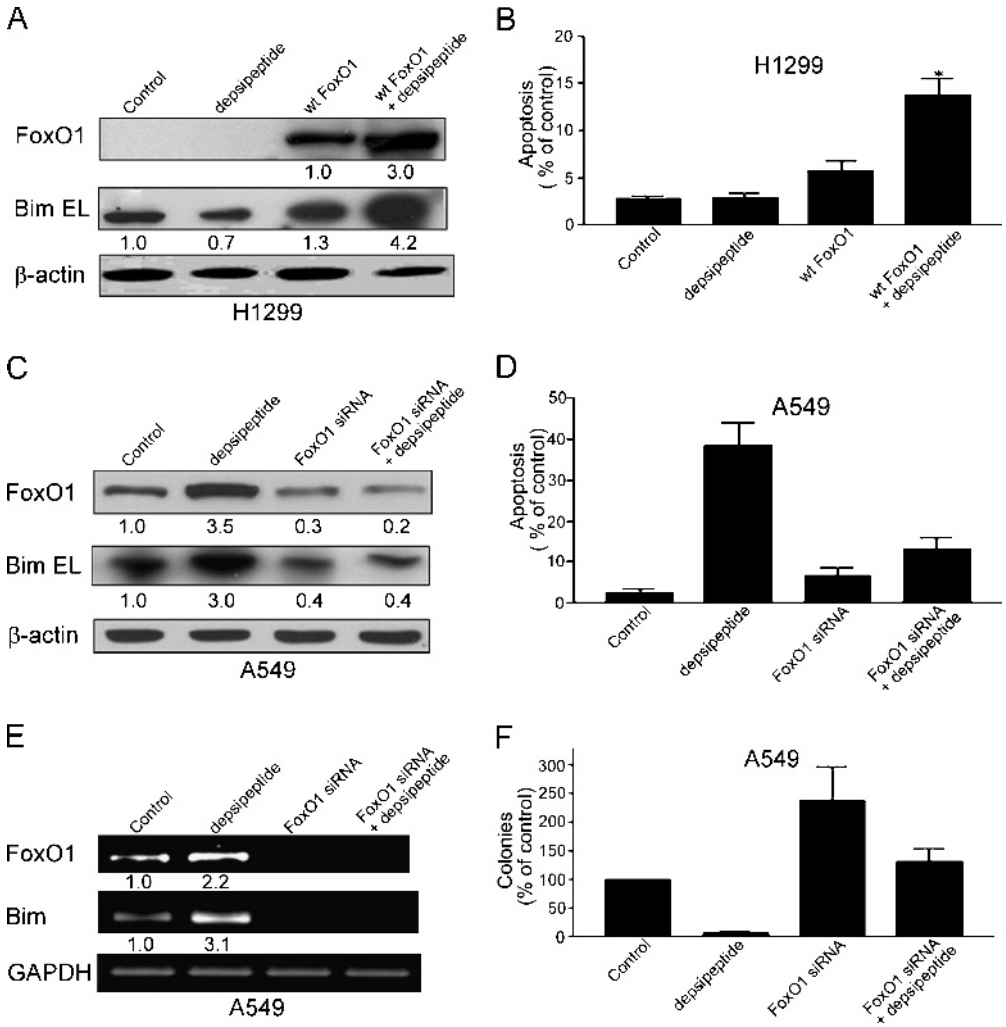

Figure 4. FoxO1 mediates Bim expression in response to depsipeptide treatment. (A) H1299 cells were treated with depsipeptide (0.05 μM for 24 hours) after transfection with wide-type FoxO1 or a nonspecific plasmid, and cells were then harvested and subjected to Western blot analysis to identify FoxO1 or Bim expression. (B) Under the same stimulated condition, cells were harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Apoptotic cells were counted and presented as a percentage of total cells. *$P < .05$. (C) A549 cells were transiently transfected with oligonucleotides of FoxO1 siRNA or a control RNAi and then treated with depsipeptide (0.05 μM for 24 hours). The cells were then harvested and subjected to Western blot analysis to identify the expression of FoxO1 and Bim. (D) Under the same stimulated condition, cells were harvested and stained with propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Apoptotic cells were counted and presented as a percentage of total cells. (E) A549 cells were transfected with a FoxO1 RNAi plasmid. Cells with knockdown of FoxO1 were treated with depsipeptide (0.05 μM for 24 hours) and then harvested to perform RT-PCR to detect FoxO1 and Bim expression. (F) A549 cells were treated with various doses of depsipeptide (0.01 to 0.1 μM) and then plated into six-well plates for colony formation. After 10 to 14 days, colonies were fixed with methanol/acetic acid, stained with crystal violet, and counted under a microscope. Results are presented as the mean ± SD of three experiments performed in triplicate for flow cytometry analysis or colony formation. For each Western blot analysis assay, β-actin is shown as a loading control.

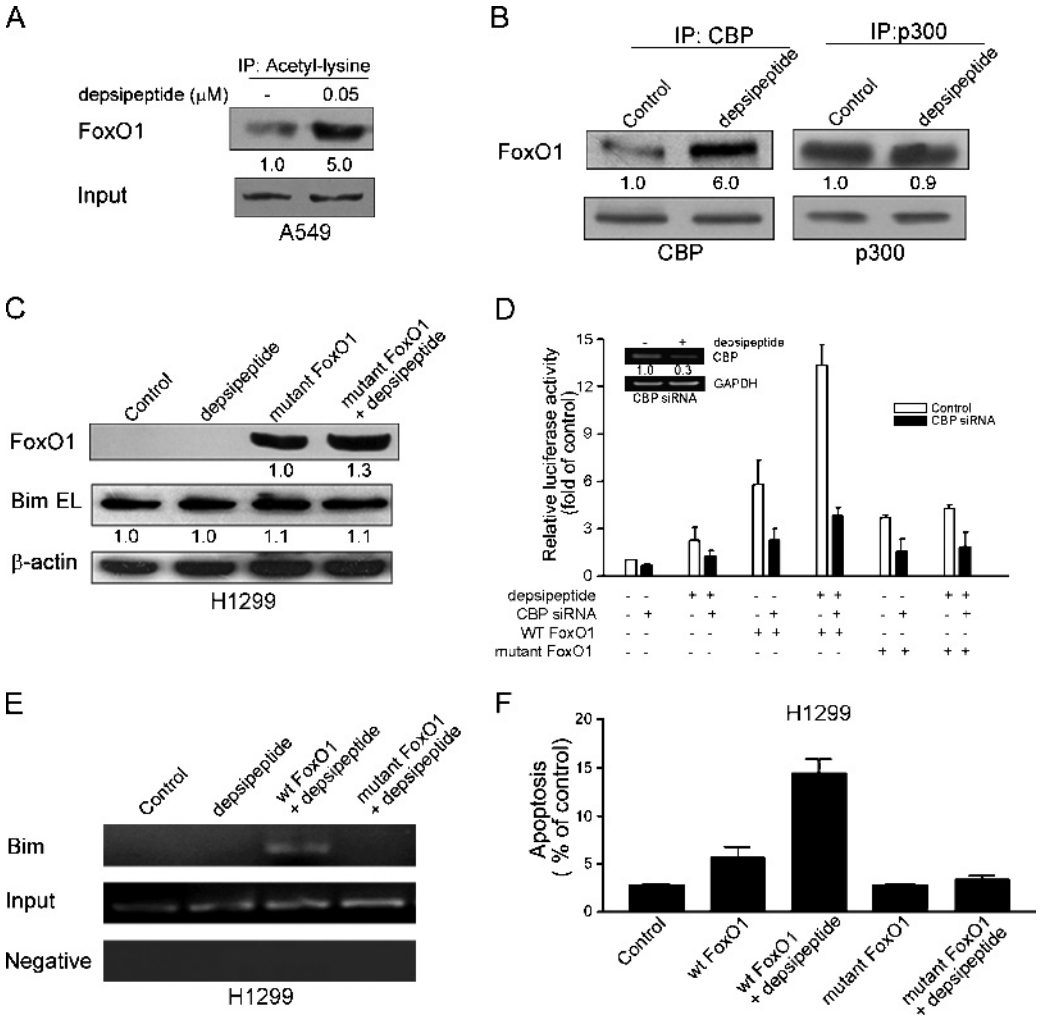

Figure 5. FoxO1 acetylation is induced by enhanced recruitment of CBP to FoxO1 and is required for depsipeptide induced Bim expression. (A) A549 cells were treated with depsipeptide (0.05 μM for 12 hours), and protein was extracted for CoIP using anti–acetyl-lysine antibody, followed by Western immunoblot analysis with anti-FoxO1. Before and after depsipeptide treatment, identical amounts of FoxO1 were used as a loading control as shown as input. (B) After depsipeptide (0.05 μM) treatment, the protein from A549 cells was extracted for CoIP with anti-CBP or p300, followed by Western immunoblot analysis with anti-FoxO1. The blots were also reprobed with CBP or p300 antibody as loading controls. (C) H1299 cells were treated with depsipeptide (0.05 μM for 24 hours) after transfection with a mutant FoxO1-3KR plasmid or a nonspecific plasmid. The cells were then harvested and subjected to Western blot analysis to detect FoxO1 or Bim expression. β-Actin is shown as a loading control. (D) H1299 cells were plated on 24-well plates after transfection with CBP siRNA or control siRNA and then transfected with Bim-pGL3 luciferase reporter construct (containing putative FoxO1-binding sites) combined with wild-type GFP-FoxO1, mutant FoxO1-3KR, or nonspecific plasmids according to experimental design. After transfection, cells were incubated in the presence or absence of depsipeptide for 12 hours, and luciferase activity was measured. Results are presented as the mean ± SD of four experiments done in triplicate. (E) H1299 cells were transfected with a wild-type FoxO1 or mutant FoxO1-3KR plasmid and were then treated with depsipeptide (0.05 μM). After 24 hours, cells were harvested for ChIP assay with a special sequence of the *Bim* promoter by using anti-FoxO1. The bands with anti-IgG served as negative controls. (F) H1299 cells were treated with depsipeptide (0.05 μM for 24 hours) after transfection with wild-type FoxO1 or mutant FoxO1-3KR plasmid, harvested, and stained by propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Results are presented as the mean ± SD of three experiments performed in triplicate for flow cytometry analysis. Protein bands were scanned by phosphorimaging, and the relative band intensities are shown as mentioned above.

in Figure 5*C*, neither did depsipeptide induce any obvious increase in expression of mutated FoxO1 nor was expression of Bim increased in response to depsipeptide treatment. To further confirm the operation of this mechanism, a luciferase assay was performed to analyze transcriptional activity of FoxO1 on the Bim promoter. H1299 cells were transfected with CBP siRNA or control RNAi and were then transfected with a Bim-pGL3 luciferase reporter construct combined with wild-type GFP-FoxO1 or mutant FoxO1-3KR. After this treatment, cells were incubated in the presence or absence of depsipeptide at 0.05 μM for 12 hours, and relative luciferase activity was measured. The relative luciferase activity of Bim was increased 13.3-fold in cells with wild-type FoxO1 transfected in response to depsipeptide treatment compared with that in control H1299 cells (Figure 5*D*). However, relative luciferase activity was not significantly increased after depsipeptide treatment in cells transfected with mutant FoxO1-3KR (Figure 5*D*). It was of interest that the increase in relative luciferase activity induced by

depsipeptide treatment in the wild-type FoxO1-transfected cells was significantly reduced when the cells were pretransfected with CBP-RNAi (5.6-fold increase in relative luciferase activity). These data indicated that depsipeptide induced *Bim* transcriptional activity through FoxO1 acetylation.

In addition, a chromatin immunoprecipitation (ChIP) assay was performed to determine whether acetylated FoxO1 directly binds to the *Bim* promoter. Figure 5*E* showed that binding of FoxO1 to the *Bim* promoter was significantly increased when cells were treated with depsipeptide. However, mutated FoxO1 was not able to bind to the *Bim* promoter. These data were consistent with the results of depsipeptide-induced apoptosis in H1299 cells (Figure 5*F*), in which depsipeptide did not induce apoptosis in the H1299 cells transfected with FoxO1-3KR, suggesting that FoxO1 acetylation resulting in the recruitment of CBP is required for the depsipeptide-induced apoptosis.

Depsipeptide Also Acetylates FoxO1 Partly through FHL2-Dependent Decreased Binding of SIRT1 to FoxO1

In addition to histone acetylase activity, some transcription factors such as SIRT1 exhibit deacetylase activity, which has been reported to be involved in regulating the acetylation status of FoxOs [27,28]. It is of interest that although SIRT1 mRNA was not obviously changed after depsipeptide treatment in A549 cells (Figure 6*A*), the interaction between FoxO1 and SIRT1 was reduced in a dose-dependent manner after depsipeptide treatment as determined by CoIP (Figure 6*B*), suggesting that SIRT1 may participate in a depsipeptide-induced acetylation of FoxO1 in A549 cells. To further confirm this hypothesis, the SIRT1 inhibitor nicotinamide was used to test the effect of SIRT1-involved acetylation of FoxO1. The mRNA level of Bim was significantly increased in A549 cells when cells were treated with nicotinamide (5 mM for 24 hours; Figure 6*C*). Similarly, a three-fold increase in FoxO1 acetylation was also observed in nicotinamide-treated cells, although there was no change in the mRNA level of FoxO1 in nicotinamide-treated A549 cells (Figure 6, *C* and *D*). These data showed that SIRT1 is involved in regulating the acetylation status of FoxO1 and thus, mediates the expression of Bim in A549 cells. Next, we investigated which factor induces a decrease in the interaction of FoxO1 and SIRT1 in response to depsipeptide treatment, and several SIRT1-related proteins were investigated. Among the proteins tested, FHL2 was significantly decreased in response to depsipeptide treatment in A549 cells (Figure 6*E*), and this led us to consider whether there might be a link between decreased FHL2 and acetylation of FoxO1. Using RNAi against FHL2 in A549 cells, we observed that the interaction of SIRT1 with FoxO1 was significantly decreased in association with an increase in FoxO1 acetylation in these cells, although the total amount of SIRT1 remained unchanged (Figure 6, *F*–*G*). These data were consistent with the results of depsipeptide-induced apoptosis in FHL2 RNAi A549 cells (Figure 6*H*), in which FHL2 siRNA synergized the effect of depsipeptide in inducing apoptosis in the A549 cells. Therefore, FoxO1 acetylation may be also mediated through an FHL2-dependent decrease in binding of SIRT1 to FoxO1 in response to depsipeptide treatment.

Discussion

Depsipeptide has been reported to induce apoptosis in different types of cancer cells by multiple pathways including activation of Bim [45]. However, the exact mechanisms underlying this effect are not well understood. Our results here demonstrate an additional novel mechanism by which the HDAC inhibitor depsipeptide induces apoptosis in cancer cells through a FoxO1 acetylation-Bim pathway.

In fact, attention has begun to be paid to induction of apoptosis by pathways involving interaction of FoxO family proteins and Bim. For example, the FoxO3-Bim pathway has been reported to play a role in inducing apoptosis in several tumor cell lines in response to various stimuli [24,58,59]. In addition, FoxO4 was also reported to activate the *Bim* promoter and thus induce a FoxO4-dependent apoptosis [26]. In this study, our data clearly show that FoxO1 is involved in Bim-regulated apoptosis in response to depsipeptide treatment (Figure 4). The FoxO1/Bim pathway induced by depsipeptide seems to be unique because other HDAC inhibitors such as SAHA and apicidin did not induce obvious changes in FoxO1 or Bim, although apoptosis was also partly induced in A549 cells in response to SAHA or apicidin (Figures 1*D* and 3*E*). SAHA can effectively induce cell cycle arrest (data not shown) instead of apoptosis in A549 cells (Figure 1*D*), although SAHA was reported to upregulate the expression of Bim in human multiple myeloma cell lines [43]. This distinct role played by SAHA in activating Bim expression may result from a difference in the tumor cell lines used. Therefore, our data extend understanding of the mechanistic link between FoxO1 and Bim in response to depsipeptide treatment.

We further explored the novel concept link that FoxO1 acetylation is required for activation of Bim. Posttranslational modification of FoxOs is a well-identified mechanism for regulating gene expression, which includes phosphorylation, ubiquitination, and acetylation [14,18,60]. Among the recognized regulators of FoxO phosphorylation, the primary regulation signal for FoxOs was the PI3-K/Akt signaling pathway [18,21,60]. FoxO1 was reported to be phosphorylated at three sites (Thr-24, Ser-256, and Ser-319) in a PI3-K–dependent manner, and the phosphorylation of all these sites or a subset thereof contributes to the inactivation of its transcriptional activity [18,21,23,60]. In our data, phosphorylation of FoxO1 was found not to be important in the depsipeptide-induced apoptosis in A549 cells (data not shown). This phosphorylation independent FoxO1 activation may result from the depsipeptide's ability to induce an increase in the expression of phosphatase and tensin homolog deleted on chromosome ten (data not shown), which antagonizes the PI3-K/AKT–dependent signaling pathway [61]. In addition, acetylation of the FoxOs was also reported to play a key role in activating downstream targets of FoxO1 [27,28]. FoxOs can interact with proteins such as CBP, p300 [62,63], which have histone acetyltransferases activities in response to oxidative stress and thus induce increased acetylation of FoxOs [27,28]. Conversely, HDACs such as SIRT1 have been reported to interact with and deacetylase FoxO proteins [27–29]. For example, SIRT1 was reported to deacetylate and represses the activity of FoxO3a [27]. In this study, there are two possible pathways through which FoxO1 acetylation may have been induced. First, FoxO1 acetylation may have been induced by the recruitment of CBP to FoxO1 directly (Figure 5*B*). Our data were consistent with the finding that FoxO1 was acetylated in HepG2 cells by the p300/CBP complex when treated with glucocorticoids and insulin [62]. In addition, CBP was reported to interact with and acetylate FoxO1 in mouse cells *in vitro* and *in vivo* [63]. Second, FoxO1 acetylation might be mediated indirectly by FHL2 in depsipeptide-treated A549 cells. The obvious decrease in FHL2 mRNA was accompanied by a reduced interaction between SIRT1 and FoxO1 after depsipeptide treatment (Figure 6, *B* and *E*). Also, FoxO1 acetylation was found to be increased in FHL2 siRNA–transfected A549 cells (Figure 6*G*), showing that FHL2 was involved in decreasing FoxO1 acetylation in A549

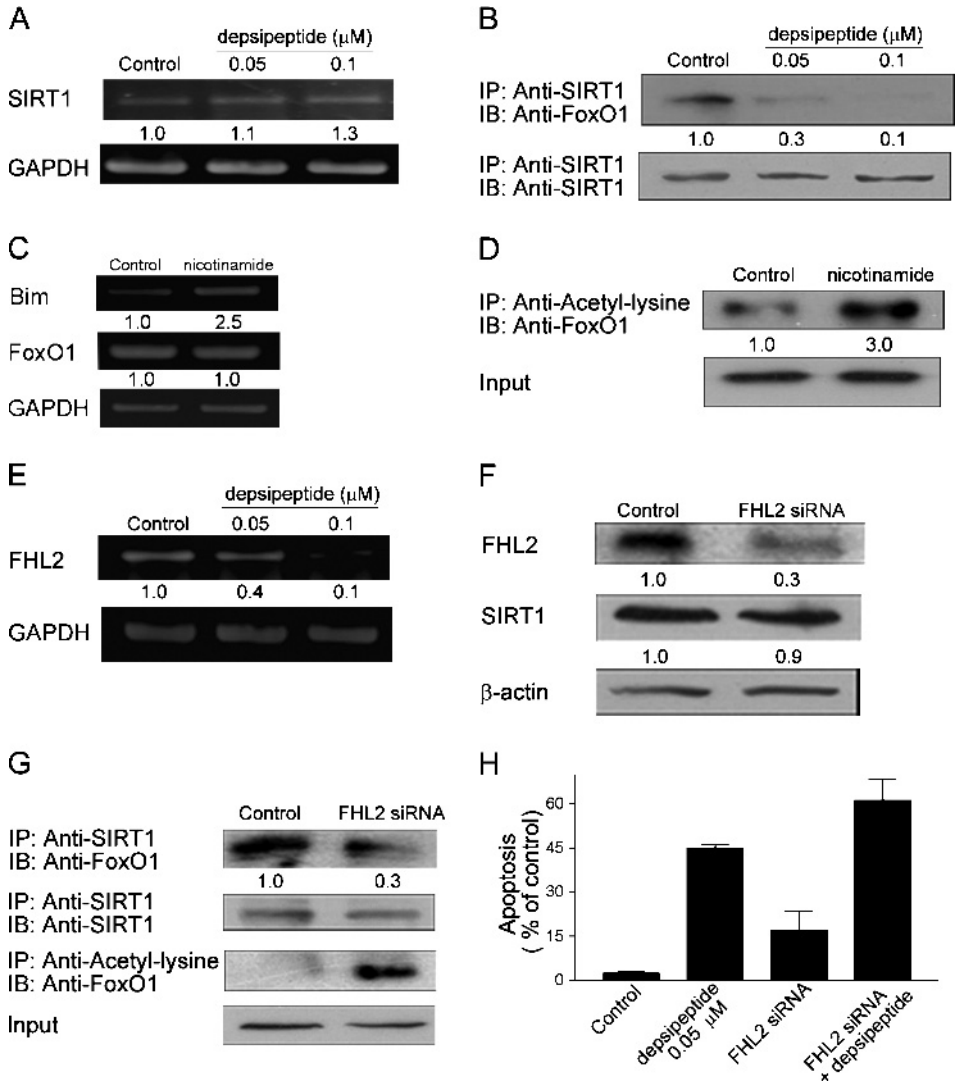

Figure 6. Depsipeptide also acetylates FoxO1 through FHL2-dependent decreased binding of SIRT1 to FoxO1. (A) A549 cells were treated with depsipeptide (0.05 to 0.1 μM for 24 hours). Cells were then harvested and subjected to RT-PCR to detect the expression of SIRT1. GAPDH was used as a loading control for RT-PCR. (B) After depsipeptide (0.05 to 0.1 μM) treatment, the protein form A549 cells was extracted for CoIP with anti-SIRT1, followed by Western immunoblot analysis with anti-FoxO1. The blots were reprobed with SIRT1 antibody as a loading control. (C) A549 cells were treated with nicotinamide (5 mM for 24 hours). Cells were then harvested and subjected to RT-PCR to detect the expression of Bim and FoxO1. GAPDH was used as a loading control for RT-PCR. (D) A549 cells were treated with nicotinamide (5 mM for 24 hours), and protein was extracted for CoIP using anti–acetyl-lysine antibody, followed by Western immunoblot analysis with anti-FoxO1. Before and after depsipeptide treatment, identical amounts of FoxO1 were used as a loading control (input). (E) A549 cells were treated with depsipeptide (0.05 to 0.1 μM for 24 hours). The cells were then harvested and subjected to RT-PCR to detect the expression of FHL2. GAPDH was used as a loading control for RT-PCR. (F) A549 cells were transfected with either oligonucleotides of FHL2 siRNA or a control RNAi, and protein was then extracted for Western blot analysis to detect the expression of FHL2. β-Actin is shown as a loading control. (G) At the same stimulated condition, cells were harvested for CoIP by using anti–acetyl-lysine and SIRT1 antibody, followed by Western immunoblot analysis with anti-FoxO1. As a control, identical amounts of FoxO1 were loaded as shown in input. (H) A549 cells were treated with depsipeptide (0.05 μM for 24 hours) after transfection either with oligonucleotides of FHL2 siRNA or with a control RNAi, harvested, and stained by propidium iodide (10 μg/ml) to determine their DNA profiles by flow cytometry. Results are presented as the mean ± SD of three experiments performed in triplicate for flow cytometry analysis. Protein and mRNA bands were scanned by phosphorimaging, and the relative band intensities are shown as mentioned above.

cells. Thus, depsipeptide-induced decrease in FHL2 expression may indirectly lead to the acetylation of FoxO1 through a decreased association between SIRT1 and FoxO1.

Finally, we explored the effect of acetylation on the activation of FoxO1. Acetylation of FoxOs has been shown to have an active role in inducing downstream gene expression, although debate about acetylation-associated activation continues [28,62–65]. For example, FoxO1 acetylation was found to increase FoxO1 stability and thus protected against pancreatic β cell failure through NeuroD and MafA induction [64]. Acetylation also enhanced FoxO3 transcriptional activation and increased the expression of Bim [28]. Conversely, acetylation of FoxO1 was reported to attenuate its transcriptional activity [63,65]. In this study, we found that acetylation of FoxO1 could increase Bim's transcriptional activity in A549 cells in response to depsipeptide

treatment. FoxO1 mutated at acetylation sites exhibited a reduced ability to bind to the *Bim* promoter and a decreased apoptosis in response to depsipeptide treatment (Figure 5, *E* and *F*), indicating that depsipeptide-induced apoptosis mediated by Bim is modulated through the FoxO1 acetylation. In addition, the expression of other downstream targets of FoxO1 including G6Pase, MnSOD, GADD45, and p27 was investigated in this study. Because the endogenous expression of G6Pase and MnSOD in A549 cells was not detectable (data not shown), thus the mRNA level of p27 and GADD45 in A549 cells in response to depsipeptide treatment was determined. In distinction to GADD45, both p27 mRNA and binding of FoxO1 to the p27 promoter were reduced in A549 cells after depsipeptide treatment (Figure W1, *A* and *B*), which is consistent with previous reports [63]. These results reflect the fact that depsipeptide-induced acetylation of FoxO1 is a unique modification that activates Bim expression, through which apoptotic cell death is elicited.

In summary, we identified a novel pathway, FoxO1/Bim, which activates apoptotic cell death in response to depsipeptide treatment. Understanding the role of acetylation of FoxO1 on its downstream activation targets will likely be useful in designing more effective therapeutic strategies for the treatment of cancer.

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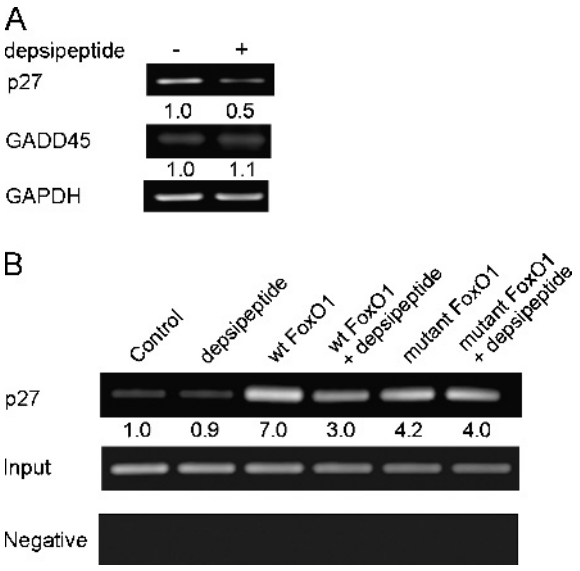

Figure W1. Depsipeptide reduces expression of p27. (A) A549 cells were treated with depsipeptide (0.05 μM for 24 hours). Cells were then harvested and subjected to RT-PCR to detect expression of p27 and GADD45. GAPDH was used as a loading control for RT-PCR. (B) H1299 cells were transfected with a wild-type FoxO1 or mutant FoxO1-3KR plasmid and then treated with depsipeptide (0.05 μM). After 12 hours, cells were harvested for ChIP assay with a special sequence of the *p27* promoter by using anti-FoxO1. The bands with anti-IgG served as negative controls. The numerical values of ChIP signal represent the percentages of input. mRNA bands were scanned by phosphorimaging, and the relative band intensities are shown as mentioned above.